Time flies: Time of day and social environment affect cuticular hydrocarbon sexual displays in *Drosophila serrata* by Susan N. Gershman, Ethan Toumishey, and Howard D. Rundle
Knowledge Bank http://hdl.handle.net/1811/61482

24H

Virgin adults were collected at emergence using light CO_2 anaesthesia, separated by sex, and housed in groups of eight individuals per vial. Starting at the beginning of the light cycle on the fourth day after emergence, CHCs were extracted hourly for 24 h. Each hour, extractions were performed on 16 males and 16 females, with four individuals randomly sampled from each of four housing vials for each sex (discarding the remaining individuals in these vials). CHCs were extracted as previously described. To ensure that individuals sampled during the dark phase of the cycle were not exposed to light, at the beginning of the dark cycle all housing vials were wrapped in aluminium foil and plugged with a dense cotton plug. To extract CHCs, the cotton plug was pierced with a wide-bore needle and CO_2 was introduced into the vial at a high flow rate to rapidly anaesthetize the flies. Only after flies were unconscious were they removed from the dark vial, at which point their CHCs were extracted within seconds.

The resulting samples were analysed via gas chromatography. After integration, to correct for technical error associated with quantifying absolute abundances, relative abundances were calculated separately for each individual by dividing the area integrated for each of their CHCs by the total area for all nine CHCs. To break the unit-sum constraint inherent in such compositional data, proportions were transformed into eight logcontrast values, using Z,Z-5,9-$C_{24:2}$ as the common divisor, following past studies on this species. We used the Mahalanobis distance technique in the multivariate analysis procedure of JMP v. 9.02 (SAS Institute, Cary, NC) to remove a small number of multivariate outliers, likely representing integration errors or contaminated samples.

To determine multivariate CHC attractiveness, individuals were scored using the vector of sexual selection gradients (***β***) calculated from an independent set of binomial choice mating trials. These mating trials were conducted separately for each sex. This scoring generated individual values of the single trait (i.e. linear combination of logcontrast CHCs) that was most strongly associated with mating success in both males (CHCβ_{males}) and females (CHC$\beta_{females}$), interpreted as their CHC-based attractiveness to the opposite sex.

Raw data column labels

label: The sample names generated by the authors (sex/time of day/vial number).
column: F = CHC samples run on the front column of the gas chromatography machine;
B = CHC samples run on the back GC column
sex: M = male; F = female
time: time of day using a 24-hr clock
vial: 4 vials were sampled for each interval of time. Vial=identification number for each vial
chc1: The integrated area under the GC peak for (Z,Z)-5,9-$C_{24:2}$
chc2: The integrated area under the GC peak for (Z,Z)-5,9-$C_{25:2}$
chc3: The integrated area under the GC peak for (Z)-9-$C_{25:1}$
chc4: The integrated area under the GC peak for (Z)-9-$C_{26:1}$
chc5: The integrated area under the GC peak for 2-Me-C_{26}
chc6: The integrated area under the GC peak for (Z,Z)-5,9-$C_{27:2}$
chc7: The integrated area under the GC peak for 2-Me-C_{28}
chc8: The integrated area under the GC peak for (Z,Z)-5,9-$C_{29:2}$
chc9: The integrated area under the GC peak for 2-Me-C_{30}

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24H SOCIAL

Virgin adults were collected at emergence using light CO_2 anaesthesia, separated by sex, and then housed in vials in one of four social treatment groups: one male alone, six males together, one male with five females, or six males with five females. Starting at the beginning of the light cycle on the fourth day after emergence, we extracted CHCs hourly from ten males from each social environment each hour for a total of 24 h. For the two treatments with multiple males, CHCs were extracted from two males/vial, with the remaining individuals discarded. Extractions were performed, the resulting samples analysed, and logcontrast trait values calculated and scored as described above.

Raw data column labels

label: The sample names generated by the authors (id/treat/time of day).
treat: Social environment. M = 1 male alone; MF = 1 male and 5 females; MM = 6 males; MMF = 6 males and 5 females
time: time of day using a 24-hr clock
vial: identification number for each vial. In the "M" and "MF" treatments, one male was removed from each of 10 vials. In the "MM" and "MMF": treatments, 2 males were removed from each of 5 vials.
chc1: The integrated area under the GC peak for (Z,Z)-5,9-$C_{24:2}$
chc2: The integrated area under the GC peak for (Z,Z)-5,9-$C_{25:2}$
chc3: The integrated area under the GC peak for (Z)-9-$C_{25:1}$
chc4: The integrated area under the GC peak for (Z)-9-$C_{26:1}$
chc5: The integrated area under the GC peak for 2-Me-C_{26}
chc6: The integrated area under the GC peak for (Z,Z)-5,9-$C_{27:2}$
chc7: The integrated area under the GC peak for 2-Me-C_{28}
chc8: The integrated area under the GC peak for (Z,Z)-5,9-$C_{29:2}$
chc9: The integrated area under the GC peak for 2-Me-C_{30}

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LOCOMOTION

Virgin adults were collected at emergence using light CO_2 anaesthesia, separated by sex, and housed in groups of either seven males or ten females per vial. Four days after emergence, the locomotor activity of individual flies was measured using a DAM 2 activity monitor (Trikinetics, Waltham, MA, USA). The monitor uses an infrared beam to measure the number of times that a single fly, housed in a 5 x 65 mm polycarbonate tube, crosses the midpoint of the tube. We programmed the DAMSystem Collection software (Trikinetics, Waltham, MA, USA) to sum activity over 10 min periods. We simultaneously used three arrays to measure the separate activity of 43 females, 44 males, and two empty tubes as negative controls, with the arrays set up as described in Charette *et al*. The tubes contained a non-nutritive 2% agarose medium for moisture. Males and females were visually separated from one another by cardboard dividers. Flies were lightly anaesthetized with CO_2, transferred into the activity monitor at 21:00 and remained in it for 48 h. Although the monitor collected all 48 h of data (see Fig. S1), statistical analyses were restricted to 24 h starting from 07:00 the morning after their introduction. This was done to allow the flies time to acclimate to their new conditions and for their activity to settle after introduction, yet to avoid possible effects of desiccation stress (by 48 h, the medium had begun to dry and pull away from the sides of the tubes).

Raw data column labels

sex: F=female; M=male
id: identifies individual males (1-44) and females (45-87).
time: 10-minute time intervals labelled as 1-144. (There are 144 10-minute time intervals in a 24-hour period.)
activity: the number of times that the individual crossed the laser at the centerpoint of the chamber during the 10-minute interval.
Light: Whether the lights in the growth chamber were on or off. 1=lights on; 0=lights off

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MATING ACTIVITY

This assay was designed to quantify sexual activity in 4-day-old males and females. Males and females were collected at emergence using light CO_2 anaesthesia and housed in mixed-sex vials of approximately 12 flies/vial where they had the opportunity to gain mating experience. Three days later, six males and six females were transferred to a 35 x 10 mm petri dish 'arena' containing a layer of non-nutritive 2% agarose medium on the bottom and sealed with Parafilm® to prevent water loss. The flies were allowed to acclimate to each other and the arena for 24 h before image collection started.

After the acclimation period, images were collected every 2 min for 24 h. Image capture was performed by a Canon Powershot G10 digital camera using Remotecapture 2.7 software (Canon U.S.A. Inc., Melville, NY) suspended above the arena on a fixed arm. Flies were not disturbed during the acclimation or data collection periods. During the 12 h dark phase, all external light was blocked and the arena was lit by 830 nm wavelength infrared lights. Previous research suggests that *Drosophila* are insensitive to light above approximately 650 nm. Images were captured from only one arena at a time. Over a 51 d period, 51 cohorts of flies were reared to emerge on consecutive days, and 51 replicate arenas were observed. All individuals were 4 day-old adults at the time of observation, and no individual was ever observed in more than one replicate arena.

All images were examined by a human observer (SNG) to score all instances in which a fly was observed mounting another in a configuration consistent with copulation. If the same pair of flies remained in copula for at least two successive images, this was scored as a mating because previous studies indicate that a *D. serrata* male must remain mounted for at least 157 sec for successful sperm transfer. Because individual flies could not be identified, the number of matings may underestimate the true number (i.e. if, between images, one pair stopped copulating and another started), although such an effect is likely small given the observed mating rate relative to the short time interval between images. Copulations that were observed in only a single image were classified as 'mounts'. We know from previous observations that *D. serrata* males will occasionally mount other males, although these are generally brief (< 20 sec; S. Gershman and H.D. Rundle, pers. observation). It was not possible to determine from the captured images whether the mounted fly was a male or a female, and mounts therefore includes both unsuccessful male-female copulations as well as male-male mounts. Summing the number of matings and mounts provides a measure of total mating activity. Because mating rates were low, observations were grouped into 24 1 h intervals, comparable to the CHC data below, summing all occurrences within an arena in a given hour.

Raw data column labels

Time: The hour of behavioural observation. The first two digits of each 4-digit number represent the hour in 24-hour time, and the second two digits represent the minutes. For example, "0700-0759" represents the mating activity that occurred between 7:00 and 7:59 am.
Day: the date of the 24-hour period in which the mating activity was monitored.
Matings: the numbers of mounted flies that persisted for two or more minutes, suggesting successful sperm transfer.
Mounts: the numbers of mounted flies that persisted for less than two minutes, indicating that sperm transfer was not successful.
Total: the total number of successful matings and unsuccessful mounts, indicating overall mating activity.